The Soluble Acyl-Acyl Carrier Protein Synthetase of *Vibrio harveyi* B392 Is a Member of the Medium Chain Acyl-CoA Synthetase Family†

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ABSTRACT: The gene encoding the unique soluble acyl-acyl carrier protein synthetase (AasS) of the bioluminescent *Vibrio harveyi* strain B392 has been isolated by expression cloning in *Escherichia coli.* This enzyme catalyzes the ATP-dependent acylation of the thiol of acyl carrier protein (ACP) with fatty acids with chain lengths from C4 to C18. The gene (called *aasS*) encodes a protein of 60 kDa, a hexahistidine-tagged version of which was readily expressed in *E. coli* and purified in large quantities. Surprisingly, the sequence of the encoded protein was significantly more similar to that of an acyl-CoA synthetase of the distantly related bacterium, *Thermus thermophilus,* than to that of the membrane-bound acyl-acyl carrier protein synthetase of *E. coli,* an enzyme that catalyzes the same reaction from a more closely related organism. Indeed, the AasS sequence can readily be modeled on the known crystal structures of the *T. thermophilus* acyl-CoA synthetase with remarkably high levels of conservation of the catalytic site residues. To test the possible role of AasS in the fatty aldehyde-dependent bioluminescence pathway of *V. harveyi,* the chromosomal *aasS* gene of the organism was disrupted by insertion of a kanamycin cassette by homologous recombination. The resulting *aasS::kan* strains retained low levels of acyl-acyl carrier protein synthetase consistent with prior indications of a second such activity in this bacterium. The mutant strains grew normally and had normal levels of bioluminescence but were deficient in the incorporation of exogenous octanoic acid into the cellular phospholipids of *V. harveyi,* particularly at low octanoate concentrations. These data indicate that AasS is responsible for a high-affinity and high-capacity uptake system that efficiently converts exogenous fatty acids into acyl-ACP species competent to enter the fatty acid biosynthetic cycle.

Acyl carrier proteins (ACPs) are small (<80 residues), highly acidic proteins. ACPs are modified by covalent attachment of 4′-phosphopantetheine moiety attached via a phosphodiester linkage to the hydroxyl group of a specific serine residue located in the midst of the protein (*1*). The paradigm ACPs are those of bacterial fatty acid synthesis where the sulfhydryl group of the 4′-phosphopantetheine moiety carries the growing fatty acid chain (*1*). In most cases, fatty acid synthesis is the only source of acylated derivatives of ACP (acyl-ACPs). However, two enzymes that catalyze conversion of long chain fatty acids to ACP acyl thioesters have been described. The first example of such an enzyme was *Escherichia coli* acyl-ACP synthetase (Aas) that was demonstrated by Ray and Cronan (*2*) to catalyze the following reaction.

$$\text{fatty acid} + \text{ACP-SH} + \text{ATP} \rightarrow \text{fatty acyl-ACP} + PP_i + \text{AMP} \quad (1)$$

The reaction proceeds in two steps through an acyl-adenylate (acyl-AMP) intermediate as expected from the production of pyrophosphate and previous studies of acetyl- and acyl-CoA synthetases (*2*).

$$\text{fatty acid} + \text{ATP} \rightarrow \text{fatty acyl-AMP} + PP_i \quad (2)$$

$$\text{fatty acyl-AMP} + \text{ACP-SH} \rightarrow \text{fatty acyl-ACP} + \text{AMP} \quad (3)$$

However, the *E. coli* acyl-ACP synthetase required non-physiological salt concentrations for activity (*2*), and because chaotrophic salts were the most efficient activators (*3*), it seemed likely that the acyl-ACP synthetase activity observed was an artifact of the in vitro assay conditions. Indeed, Jackowski and co-workers (*4*) later showed this to be the case. *E. coli* Aas has been demonstrated to be a bifunctional protein having both 2-acylglycerolphosphorylethanolamine acyltransferase and acyl-ACP synthetase activities (*4*). The overall function of the enzyme is to salvage the 2-acylglycerolphosphorylethanolamine produced during lipoprotein acylation by its conversion back to phosphatidylethanolamine. In a tightly coupled reaction, the enzyme first forms acyl-ACP and then transfers the acyl group to 2-acylglycerolphosphorylethanolamine. Therefore, the acyl-ACPs that are synthesized are present only as intermediates that remain firmly bound to this membrane enzyme (*4*). Due to this sequestration, exogenous fatty acids are directly incorporated into phospholipids in vivo without any opportunity for elongation by the fatty acid synthetic pathway. Although its

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activity could not be used to manipulate the fatty acid synthetic pathway, *E. coli* Aas activity has long been commercially available because it is extremely valuable in the preparation of acyl-ACPs used as substrates for other enzymes of fatty acid and complex lipid synthesis in a variety of biological systems (*5*−*8*). *E. coli* Aas has also been used to detect and assay ACP levels in diverse biological systems (*9*, *10*).

The second acyl-ACP synthetase is that reported by Byers and co-workers in the bioluminescent bacterium *Vibrio harveyi* (*11*−*14*). This is a soluble enzyme that catalyzes acyl-ACP synthesis using the same mechanism as the acyl-ACP synthetase activity of *E. coli* (*12*). These workers searched for this enzyme because the light production (luciferase) reaction produces tetradecanoic acid that fails to accumulate (*15*). Moreover, unlike *E. coli*, *V. harveyi* elongates exogenously supplied fatty acids which requires their conversion to acyl-ACPs (*16*). These investigators demonstrated the enzyme activity, its lack of coordinate regulation with the bioluminescence enzymes, and its utility in making acyl-ACPs (*12*, *14*). They then purified the enzyme to apparent homogeneity (*13*). The enzyme was reported to be a single monomeric polypeptide of 62 000 Da, but no N-terminal sequence data or other data useful in isolation of the encoding gene were reported. We report the identification of the gene encoding the *V. harveyi* acyl-ACP synthetase by expression cloning. We have named the gene *aasS* for acyl-ACP synthetase-soluble. The His-tagged protein has been purified to homogeneity in milligram quantities. We have disrupted the *aasS* gene in a wild-type strain of *V. harveyi* and find that it plays no role in bioluminescence, although it does provide a high-affinity system for uptake of fatty acids from the medium.

EXPERIMENTAL PROCEDURES

Materials. The CopyControl Fosmid Library Production Kit for construction of the cosmid expression library, End-Repair Enzyme Mix, Colony Fast-Screen Kit (Size Screen), and MaxPlax Lambda Packaging Extracts were all purchased from Epicentre (Madison, WI). The Large-Construct Kit was purchased from Qiagen (Valencia, CA). The λCE6 phage stock and its host strain, LE392, were purchased from Novagen (Madison, WI). The labeled fatty acids were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Genomic DNA of *V. harveyi* M17 was a gift from C.-Y. Lai of this laboratory. Acyl-ACP synthetase partially purified from *V. harveyi* according to the method of Shen et al. (*14*) was obtained from K. Janiyani of this laboratory. Purified *E. coli* holo-ACP (*17*, *18*) was a gift from J. Solbiati of this laboratory. The *cis*-3-decenoic acid was a gift of Wacker Chemie (Munich, Germany). DNA sequencing was performed by the Biotechnology Center, University of Illinois. Bioluminescence was measured at 380−630 nm using a Molecular Devices LMax11^{384} microplate reader in the Carver Metabolomics Center, University of Illinois.

Bacterial Strains and Plasmids. Bacterial strains and plasmids that were used are listed in Table 1. Wild-type *V. harveyi* strains B392 and M17 were obtained from D. Byers (*12*). The *aasS* gene was isolated from the genome of strain M17, a dark mutant of strain B392, used in prior studies of AasS (*12*−*14*). This strain lacks a bioluminescence-related fatty acyl esterase activity and only emits light in the presence of exogenous aldehydes or fatty acids (*12*). *E. coli* host strains EP1300 and YFJ115 used for cosmid expression library construction were described previously (*19*). WM3064 is an *E. coli* donor strain used for conjugative transfer of DNA into diverse Gram-negative bacteria (*20*). Plasmid pJQ200 (*21*) was the vector used in the construction of an *aasS* deletion mutant of *V. harveyi*. This gentamycin-resistant plasmid carries both ori_{15a} and $oriT_{RP4}$ and thus can be readily replicated in *E. coli* and conjugatively transferred to recipient strains. Plasmid pJQ200 also carries the *sacB* sucrose sensitivity gene that allows counterselection against maintenance of the plasmid.

Isolate VS1−11 (subsequently designated strain YFJ155) was the strain isolated from cosmid library screening that contained AasS activity. The cosmid of this strain was named pYFJ48. Plasmid pYFJ48 was reintroduced into strain EP1300 to eliminate the pCY598 plasmid as described previously (*19*), resulting in strain YFJ264. The three clones isolated by screening the pYFJ48 subclone library were designated YFJ215, YFJ217, and YFJ219. The *aasS* gene-containing plasmids from the three strains were designated pYFJ63, pYFJ64, and pYFJ65, respectively. To construct pYFJ51 (also called pSUBlue-1), a low-copy number expression vector with blue-white screening, a 892 bp fragment of pETBlue-1 containing the multiple cloning site (MCS) together with the upstream T7 promoter and the downstream T7 terminator was PCR amplified using primers pETBlue-1 UP (5′-GAAGCACTTCAATTGTGAGCGCTCACAATTCTCGTGA-3′) and pETBlue-1 DOWN (5′-GAAGTGCTTCGGTTATGCTAGTTATTGCT-3′) to give a fragment having XmnI sites at both ends. The PCR product was cloned into pCR2.1-TOPO, resulting in pYFJ50. The MCS fragment in pSU19 (*22*) was excised from the vector by HaeII digestion and the following self-ligation of the remaining fragment, resulting in pYFJ49. pYFJ50 was digested with XmnI, and the 902 bp fragment was ligated to pYFJ49 digested with the same enzyme to give plasmid pYFJ51.

The *aasS* gene was PCR amplified from pYFJ64 using primers AAS NdeI (5′-CAT ATG AAC CAG TAT GTA AAT GAT CCA-3′) and AAS BamHI (5′-GGA TCC TTA CAG ATG AAG TTT ACG CAG T-3′). The PCR product was cloned into vector pCR2.1-TOPO, resulting in pYFJ79. The *aasS* NdeI−BamHI fragment of pYFJ79 was subsequently inserted into the same sites of pET16b, resulting in pYFJ84. Plasmid pYFJ84 was transformed into strain BL21-(λDE3), and the resulting strain YFJ239 was used for overexpression and purification of an N-terminally His-tagged form of AasS.

Plasmid pCY742 was constructed for generation of an *aasS* null mutant of *V. harveyi*. First, an 855 bp SmaI fragment containing the Tn*903*-derived kanamycin resistance cassette of p34S-Km3 (*23*) was inserted into the unique SmaI site of the *aasS* open reading frame of a kanamycin-sensitive derivative of pYFJ79 (made by PstI digestion followed by recircularization), resulting in pCY742. The 2636 bp XhoI−SpeI fragment of pCY742 containing the disrupted *aasS* gene was ligated to pJQ200 cut with the same two enzymes, resulting in pCY743. Plasmid pCY743 was transformed into *E. coli* strain WM3064, and the resulting strain was used as the donor in conjugative transfer of the plasmid into

Table 1: Bacterial Strains and Plasmids

strain or plasmid	relevant characteristics	source or reference
E. coli strain		
EP1300	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ*80d *lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galK* l⁻*rpsL trfA*	Epicentre
LE392	F⁻ *hsdR514* (r_K^-, m_K^+) *mcrA supE44 supF58 lacY1* or Δ(*lacIZY*) *galK2 galT22 metB1 trpR55*	Novagen
TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ*80d *lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK rpsL nupG*	Invitrogen
TOP10F′	F′ *lacI*^{q}Tn*10* of TOP10	Invitrogen
BL21(*λ*DE3)	F⁻ *gal hsdS λ*DE3	*51*
WM3064	RP4−2-Tc::Mu *aph*::Tn7 *dapA recA*	*20*
YFJ115	EP1300/pCY598	*19*
YFJ155	AasS-positive isolate VS1−11 isolated from the cosmid library screening; contains cosmid clone pYFJ48 in YFJ115	this work
YFJ264	EP1300/pYFJ48	this work
YFJ215	AasS-positive subclone 59 from the pYFJ48 library	this work
YFJ217	AasS-positive subclone 83 from the pYFJ48 library	this work
YFJ219	AasS-positive subclone 89 from the pYFJ48 library	this work
V. harveyi strain		
B392	wild-type *V. harveyi*	D. Byers (*11*)
M17	a dark mutant of B392 used as the DNA source in cosmid library construction	D. Byers (*11*)
CY1719	*aasS::Km3* disruption derivative of B392	this work
CY1723	*aasS::*Ω*Km* disruption derivative of B392	this work
CY1724	*aasS::*Ω*Km* disruption derivative of B392	this work
plasmid		
pCR2.1-TOPO	TOPO TA cloning vector	Invitrogen
pET16b	expression vector with a T7 promoter	Novagen
pCC1FOS	cloning vector for CopyControl Fosmid Library construction	Epicentre
pETBlue-1	blue-white screening expression vector	Novagen
pSU19	low-copy number cloning vector, chloramphenicol-resistant	*22*
pYFJ51	blue-white screening cloning and expression vector	this work
pYFJ48	*aasS* containing a cosmid clone from YFJ155	this work
pYFJ63	plasmid from YFJ215, which contains *aasS*	this work
pYFJ64	plasmid from YFJ217, which contains *aasS*	this work
pYFJ65	plasmid from YFJ219, which contains *aasS*	this work
pYFJ79	*aasS* PCR-amplified and cloned into pCR2.1-TOPO (NdeI and BamHI)	this work
pYFJ84	*aasS* cloned into the NdeI and BamHI sites of pET16b	this work
pNRD70	*A. aeolicus* ACP gene cloned in pBAD322	N. DeLay and J. E. Cronan, manuscript in preparation
pNRD71	*Ba. subtilis* ACP gene cloned in pBAD322	
pNRD73	*L. lactis* ACP gene cloned in pBAD322	
pNRD75	*B. taurus* mitochondrial ACP gene cloned in pBAD322	
pNRD136	*Ba. subtilis sfp* cloned in pDHK29	
pBAD322	pBAD expression vector containing the complete pBR322 origin	*52*
p34S-Km3	kanamycin cassette vector	*23*
pHP45Ω-Km	kanamycin cassette vector	*24*
pJQ200	cloning vector carrying *ori15a*$_{??}$and *oriT*$_{RP4}$, containing *lacZ*α for blue-white screening, *sacB* for counterselection	ATCC (*21*)
pCY742	PstI deletion derivative carrying the Km3 cassette of p34S-Km3 in the SamI site of *aasS*	this work
pCY743	XhoI−SpeI fragment of pCY742 inserted into the same sites of pJQ200	this work
p34S-Km3	kanamycin cassette vector	*23*
pHP45Ω-Km	kanamycin cassette vector	*24*

V. harveyi. Strain WM3064 requires diaminopimelic acid for growth and carries a chromosomally integrated copy of the conjugational transfer origin of plasmid RP4. For conjugational transfer of CY743 into *V. harveyi* B392, 1 mL of each of the mid-log phase cultures was grown at 30 °C in LB supplemented with kanamycin (50 *μ*g/mL) and diaminopimelic acid (50 *μ*g/mL) or LB for the donor and recipient, respectively. One milliliter each of the donor and recipient strains were mixed and centrifuged for 10 min at full speed in a desktop minicentrifuge. The cell pellets were then resuspended in 50 *μ*L of LB medium, and the resuspended cells were spread on 47 mm diameter 0.45 *μ*m membrane filters on LB plates that contained diaminopimelic acid. The plates were then incubated at 30 °C overnight. The following day, each filter was placed in a 50 mL centrifuge tube, 5 mL of buffer was added, and the cells were eluted from the membrane by vortex mixing. Various dilutions of the mating mixtures were then plated on plates of LB medium containing 30 *μ*g/mL kanamycin, 25 *μ*g/mL gentamycin, and 100 *μ*g/mL ampicillin (*V. harveyi* is naturally resistant to *β*-lactam antibiotics). Parallel sham mating control cultures of the parental strains showed no growth on these plates. Cultures of these recombinants were then spread on LB-kanamycin/ampicillin plates containing 5% sucrose, and the plates were incubated at 30 °C for up to 2 days. The resulting colonies were then tested for the loss of gentamycin resistance. Approximately half of the colonies resistant to sucrose and kanamycin were sensitive to gentamycin, indicating loss of the vector sequences (the gentamycin-resistant colonies were presumably due to mutations that inactivated SacB). One of these colonies was saved as strain CY1719. Two additional *aasS* disruption alleles called strains

CY1723 and CY1724 were similarly constructed using the SmaI fragment of the Tn5-derived kanamycin resistance cassette of pHP45Ω-Km (which includes translation termination codons in all reading frames plus the strong Ω transcription terminator) (*24*). The Ω-Km cassette was inserted in opposite orientations in the two donor plasmids. Insertion of the kanamycin cassettes into the *V. harveyi* genome was verified by PCR analysis using the primers 5′-GAGTCACTCGCAAAGCGAACG-3′ and 5′-CGTTACTGAGTACGCAGCC-3′ which begin 50 bp upstream of the *aasS* initiation codon and 100 bp downstream of the *aasS* termination codon, respectively, followed by detailed restriction mapping of the PCR products. The three disruption stains had indistinguishable physiological and enzymatic properties.

Assay of AasS Activity. Two assays for AasS activity were used in this work. The radioactive assay was adapted from that of Shen et al. (*14*). Briefly, 20−25 μM purified *E. coli* holo-ACP and 200 μM sodium [1-^{14}C]octanoate (53 mCi/mmol) or 100 μM sodium [1-^{14}C]tetradecanoate (55 mCi/mmol), 0.1 M Tris-HCl (pH 7.8), 10 mM ATP, 10 mM $MgSO_4$, 5 mM dithiothreitol (DTT), and an AasS enzyme preparation were mixed in a 50 or 100 μL reaction system and incubated at 37 °C. The reaction mixes were then loaded onto Whatman 3MM filter disks which were washed and counted for radioactivity as described by Ray and Cronan (*2*). A positive control using AasS partially purified from *V. harveyi* crude extracts (*13*, *14*) was generally included. The second assay was a gel shift assay (*3*, *5*), and the reaction was performed essentially like the radioactive assay except that 500 μM nonradioactive octanoate or tetradecanoate replaced the radioactive substrates. The samples were analyzed on 20% polyacrylamide gels containing 0.5 M urea (*6*). Several different forms of ACP (disulfide dimer, monomer, apo, holo, and acyl) are readily separated by this gel system (*6*, *18*). DTT (1 μL of a 500 mM solution) was routinely added to each sample immediately before gel loading to cleave any ACP disulfide dimers to monomers. Two protocols were used to test the substrate specificity of AasS toward the ACPs of various species. In the first protocol, *E. coli* strain DH5α-harboring plasmids encoding different ACP species were induced with 2% arabinose (with 10 μM IPTG in the case of *Lactococcus lactis* ACP and the *Bos taurus* mitochondrial ACP so that Sfp expression from plasmid pNRD136 was also induced). The cell pellets were resuspended in buffer A [20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, and 10 μM DTT] (*14*) and disrupted by sonication. Fifty microliters of each cell-free crude extract was used in the AasS assay with purified His-tagged AasS. A positive control was conducted with *E. coli* ACP, and a negative control was performed with the crude exact of DH5α carrying the pBAD322 vector. Reactions were both performed using either nonradioactive octanoic acid assayed by the gel shift assay (data not shown) or [1-^{14}C]octanoic acid (final concentration of 40 μM) assayed by autoradiography following gel electrophoresis. Assays of the extract of a strain overproducing *E. coli* ACP were also included as a positive control. In the second protocol, a host strain that carried both an Sfp-encoding plasmid and a *panD* mutation which permitted labeling of the ACP prosthetic group with tritiated β-alanine, a 4′-phosphopantetheine precursor. Cultures (50 mL) of each strain were prepared in arabinose minimal medium as previously described except that the medium contained 4 μM β-alanine. A 3 mL sample of each culture was then moved to a tube containing 45 μCi of β-[3-^{3}H(N)]alanine (60 Ci/mmol). The large and small cultures were then grown in parallel and harvested when growth ceased due to β-alanine limitation. Lysates were prepared, and those from the small radioactive cultures were processed by four cycles of ultrafiltration through Vivaspin 2 mL concentrators (5000 molecular weight cutoff from Vivascience) to separate protein-bound label from unincorporated label with CoA and its precursors followed by scintillation counting. This procedure gave the levels of holo-ACP in each extract. The larger nonradioactive extracts were then used at the ACP source in AasS assays carried out with 10 μM [2,2′,3,3′-^{3}H]octanoate (131 mCi/mmol). The assay was normalized using the data from the radioactive cultures such that each extract had a similar holo-ACP level.

Construction and Screening of the Cosmid Expression Library of V. harveyi Genomic DNA. Construction and screening of the cosmid library of *V. harveyi* genomic DNA fragments proceeded essentially as described previously (*19*). The *V. harveyi* cosmid expression library was plated on LB medium containing 30 μg/mL kanamycin and 12.5 μg/mL chloramphenicol. The plates were incubated at 37 °C for 48 h to permit the growth of very small colonies. A total of 500 colonies were screened. In the first round of screening, the 500 colonies were divided into ten 50-colony pools, including one pool of very small colonies (VS1), two pools of small colonies (S1 and S2), four pools of medium-sized colonies (M1−M4), and three pools of large colonies (L1−L3). Each pool was separately cultured, and an increased copy number was induced with 0.01% arabinose. The cell pellets were resuspended in freshly made buffer A [20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, and 10 μM DTT] (*14*) and disrupted by being passed twice through a French pressure cell. The cell free supernatants were assayed for AasS activity using the radioactive assay. Pool VS1, the 50-colony pool that exhibited activity in the first round of screening, was further divided into ten 5-colony pools, and each pool was induced and assayed as before. In the third round of screening, each colony of pool VS1 11−15 (the 5-colony pool that contained AasS activity) was induced and assayed. In the second and third round screenings, the gel assay was used in place of the radioactive assay.

Construction and Screening of the Subclone Library. The DNA of cosmid pYFJ48 which contained the *aasS* gene was prepared from strain YFJ264 as described previously (*19*). The cosmid DNA was fragmented by sonication, and 3−4 kb fragments were recovered as described previously (*19*). Cloning vector pYFJ51 was digested with SmaI, dephosphorylated with shrimp alkaline phosphatase (Roche Applied Science, Indianapolis, IN), and then ligated to the recovered 3−4 kb cosmid fragments. The ligation mixture was transformed into TOP10F′ (Invitrogen, Carlsbad, CA) and plated on LB medium containing 25 μg/mL chloramphenicol, 80 μg/mL 5-bromo-4-chloro-3-indolyl β-D-galactoside, and 200 μg/mL IPTG. The library plates were allowed to grow at 37 °C for 48 h. One hundred of the resulting white colonies were picked from the subclone library plates and streaked again on the same medium. These colonies were then screened for AasS activity in 10 pools of 10 colonies. The pools were cultured in LB supplemented with 0.2% maltose, 10 mM $MgSO_4$, and 25 μg/mL chloramphenicol and infected

with λCE6 phage to provide phage T7 RNA polymerase. The pools were then assayed for AasS activity by the gel shift assay. Each of the two 10-colony pools that exhibited AasS activity in the first round of screening was further divided into five two-colony pools and infected with λCE6 phage and assayed for AasS activity by the gel assay. Finally, the single colonies that expressed AasS activity were isolated from three 2-colony pools, infected with λCE6 phage, and assayed by the gel shift assay.

Expression and Purification of His-Tagged Acyl-ACP Synthetase. His-tagged AasS was expressed and purified from strain YFJ239 grown in LB medium containing 100 μg/mL ampicillin and induced with 1 mM IPTG. The His-tagged AasS was purified from the crude extract using Ni−NTA agarose (Qiagen, Valencia, CA) following the general protocol of the manufacturer. The imidazole concentrations used in lysis, wash, and elution buffers were 10, 20, and 250 mM, respectively. The fractions containing purified His-tagged AasS were dialyzed against AAS buffer [20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.002% Triton X-100] and stored at −80 °C.

Lipid Analyses. The *V. harveyi* strains were grown and labeled with 1-^{14}C-labeled short chain fatty acids essentially as described by Byers (*16*). After being labeled, the cultures were extracted by the method of Bligh and Dyer (*25*). The supernatant was resolved into a chloroform and water/methanol phase, and the chloroform phase was removed and dried under nitrogen. Traces of water were removed by azeotropic distillation first with absolute ethanol and then with benzene and absolute ethanol. The phospholipid acyl chains were then converted to fatty acid methyl esters by transesterification with sodium methoxide in methanol (this reagent does not methylate free fatty acids) (*26*). The methyl esters were extracted into hexane, and any free fatty acids were removed by partition against 0.1 M potassium bicarbonate. A sample of the fatty acid methyl ester fractions was taken for scintillation counting, and the remainder was then analyzed by argentation thin-layer chromatography on plates containing 20% $AgNO_3$ (*26*) to separate saturated and unsaturated species and by reverse phase thin-layer chromatography on KC18 plates (Whatman) in a 65:35:0.5 acetonitrile/acetic acid/water mixture to determine the lengths of the acyl chains (*27*). In some cases, the labeled lipids were loaded on a silica gel G plate and developed first in a 70:30:2 petroleum ether/ether/acetic acid mixture to the top of the plate and then a 65:25:8 chloroform/methanol/acetic acid mixture to halfway up the plate (*28*). The plates were then subjected to autoradiography followed by scraping the appropriate area of silica gel into scintillation vials. One milliliter of 70% ethanol was added to each vial to elute the lipids, and the contents of the vials were counted following addition of scintillation fluid.

RESULTS AND DISCUSSION

Isolation of the Gene Encoding AasS. The *aasS* gene was isolated by screening a *V. harveyi* cosmid expression library constructed essentially as described previously (*14*). Although the *V. harveyi* genome sequence has only been partially sequenced and the sequenced genomes of *Vibrio parahaemolyticus* (considered a close nonbioluminescent relative of *V. harveyi*) and the bioluminescent *Vibrio fisheri* are 5.0 and 4.2 Mb, respectively, it seemed that our prior cosmid screening approach could be successful. Therefore, a library of cosmid clones of ca. 40 kb fragments of the *V. harveyi* genome was constructed and introduced into *E. coli* by phage λ particles formed by DNA packaging in vitro. We screened 500 of the resulting colonies for AasS activity since this number was expected to cover the *V. harveyi* genome (*29*). The cosmid system that was used allows amplification of the copy number of the cosmids from approximately one per cell to 40−80 per cell upon addition of arabinose (*30*), thus increasing production of cosmid-encoded proteins. Using the gel shift assay, a first round of screening failed to show detectable AasS activity in any of the 50-colony pools. However, when the same pools were screened using the more sensitive radioactive assay, a low level of AasS activity was detected in pool VS1. Therefore, 50-colony pool VS1 was deconvoluted by division into successively smaller pools, and after two rounds of screening using the gel shift assay (Experimental Procedures), a single colony (VS1 11) that clearly produced AasS activity was isolated (Figure 1A) and was designated strain YFJ155. To confirm that the activity was that of the enzyme studied by Byers and co-workers (*12*−*14*), we compared the fatty acid substrate specificity of the activity encoded by *E. coli* strain YFJ155 with that isolated from *V. harveyi.* The substrate specificities of the enzymes from the two sources were essentially identical when assayed on fatty acids having chain lengths from C4 to C18. Both enzyme sources exhibited weak activity on both short (C4 and C6) and long (C18) fatty acids relative to medium chain acids (data not shown) as expected from prior work (*12*, *14*). We also found that the enzyme activities from both sources could utilize fatty acids of odd chain lengths (C7 to C15) as well as (albeit poorly) the unsaturated acid, *cis*-3-decenoic acid. From these data and the molecular weight of the protein encoded by the gene (see below), we believe the isolated gene encodes the enzyme studied by Byers and co-workers (*12*−*14*). Strain YFJ264,

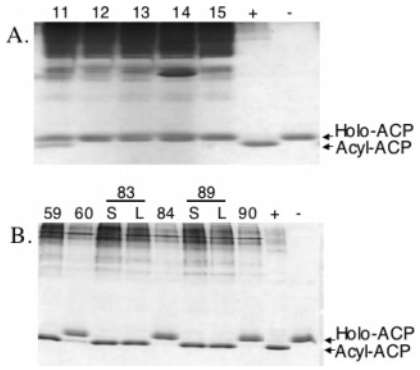

FIGURE 1: Screening of the *V. harveyi* cosmid expression library (A) and subclone library (B). Each of the five colonies of pool VS1 11−15 was individually assayed for AasS activity by the gel shift assay. A positive control (+) was conducted using partially purified AasS from *V. harveyi.* The negative control (−) was the absence of partially purified AasS. Holo-ACP and acyl-ACP (myristoyl-ACP) are shown as indicated. The weak acyl-ACP band in all lanes is presumably the acyl-ACPs formed from the *E. coli* ACP present in the crude extract. (B) Third-round screening of the subclone library. Each of the individual clones of pools 59 and 60, 83 and 84, and 89 and 90 were assayed for AasS activity. Clones 83 and 89 exhibited both small and large colonies after restreaking, and thus, both the small (S) and large (L) colonies were assayed.

which lacked T7 polymerase plasmid pCY598, also had AasS activity (data not shown), indicating that a promoter within the *V. harveyi* DNA segment was sufficiently active in *E. coli* to permit detection of AasS activity by the gel shift assay.

The *aasS* gene was isolated from the 40 kb insert of cosmid pYFJ48 by construction and screening of a library of smaller clones in pYFJ51, a low-copy number expression vector having blue-white detection for insertions into the multiple cloning site (Experimental Procedures). A library of 100 white colonies was screened, and following three rounds of screening of successively smaller colony pools, three AasS positive clones (59, 83, and 89) were isolated (Figure 1B). The three colonies were designated strains YFJ215, YFJ217, and YFJ219, respectively, whereas the plasmids they contained were designated pYFJ63, pYFJ64, and pYFJ65, respectively.

Sequences of aasS and of the Encoded Protein. All three AasS positive plasmids were sequenced using primers that annealed to the vector sequences followed by primer walking to obtain the complete sequences of each plasmid insert. The three inserts each gave a single open reading frame of 1602 bp that encoded a protein of 533 amino acids (60.4 kDa). The sequence of *aasS* has been reported to GenBank (accession number DQ525851). The deduced protein molecular mass was in good agreement with the value of 62 kDa obtained by SDS−PAGE analysis of AasS purified from *V. harveyi* crude extracts (*13*). Moreover, the open reading frame showed each of the characteristic motifs of the adenylate-forming enzyme family (*31*−*33*) and aligned with the carboxyl-terminal two-thirds of *E. coli* Aas and with several acyl-CoA synthetases of demonstrated activity (Figure 2A). Unexpectedly, the strongest match (36% identical over 492 residues) to a protein of known function was to a medium chain acyl-CoA synthetase from *Thermus thermophilus* (*32*) rather than to the enzyme that catalyzes the same reaction, *E. coli* Aas (19% identical over 486 residues). Indeed, AasS and the *T. thermophilus* protein generally exhibit the same insertions and gaps relative to the other proteins (e.g., AasS residues 339−350 with *T. thermophilus* residues 354−365). Blast searches found no close homologues of AasS in other *Vibrio* species (some of which are bioluminescent). The closest matches were to proteins of two metal-reducing δ-proteobacteria, *Geobacter sulfurreducens* (61% identical) and *Geobacter metallireducens* (60% identical).

Three high-resolution (2.2−2.5 Å) crystal structures of the *T. thermophilus* acyl-CoA synthetase were recently reported (*32*). These are the first reported crystal structures of a long chain fatty acyl-CoA synthetase and are of the apoprotein, a complex of the protein with a nonhydrolyzable ATP analogue and a complex of the protein containing the myristoyl-AMP intermediate (*32*). The last of these structures shows the mode of acyl chain binding as well as the adenylate binding determinants. Virtually all of the residues involved in ATP and myristoyl-AMP binding are conserved in *V. harveyi* AasS (Figure 3A). Indeed, of the 32 assigned active site residues of the *T. thermophilus* acyl-CoA synthetase−myristoyl-AMP complex structure, 23 are strictly conserved in AasS. Moreover, six of the nonidentical residues are very conservative substitutions (Ser for Thr, Ile for Val, etc.). Hence, it seems highly likely that the tertiary structure of AasS is very similar to that of the *T. thermophilus* acyl-CoA synthetase. Indeed, AasS was readily threaded on the *T. thermophilus* structure (http://swissmodel.expasy.org//SWISS-MODEL.html), whereas the homologous region of *E. coli* Aas failed to thread. However, the quaternary structures of the enzymes differ in that the *T. thermophilus* enzyme is a dimer (*32*) whereas AasS is a monomer (*13*). AasS was reported to be inhibited by sulfhydryl reagents, and ATP protects the enzyme from this inactivation, suggesting that a cysteine residue is located within or close to the active site (*13*). Of the four AasS cysteine residues, cysteine 268 seems to be the strongest candidate to play this role, since its analogous *T. thermophilus* acyl-CoA synthetase residue (glycine 273) is located close (<10 Å) to the active site. The protection of AasS from thiol reagents observed upon ATP binding (*13*) is consistent with the closed conformation of the *T. thermophilus* acyl-CoA synthetase engendered by ATP binding (*32*).

Despite the excellent conservation of active site residues between AasS and the *T. thermophilus* acyl-CoA synthetase (Figure 2B), the enzymes have different acyl chain length specificities. AasS has good activity with fatty acids of chain lengths from C5 to C14 but is poorly active with the longer saturated fatty acids, C16 and C18 (*12*, *14*) (data not shown), whereas the *T. thermophilus* acyl-CoA synthetase is highly active with the C16 and C18 acids but is inactive with octanoic and decanoic acids (*32*). Indeed, the *T. thermophilus* protein has only weak activity on dodecanoic acid (C12), the best of the AasS fatty acid substrates (*12*).

Expression and Purification of V. harveyi Acyl-ACP Synthetase. The putative AasS open reading frame was PCR-amplified from pYFJ64 DNA and cloned into T7 promoter expression vector pET16b, resulting in pYFJ84 that encoded an AasS derivative having an N-terminal hexahistidine tag. We tagged the N-terminus because that region of the *T. thermophilus* acyl-CoA synthetase is exposed to solution and is highly mobile (the first seven residues are not visible in the crystal structures) (*32*). Plasmid pYFJ84 was introduced into T7 RNA polymerase expression strain BL21(λDE3). T7 RNA polymerase synthesis was induced, and crude cell extracts of the induced cells were prepared and assayed for AasS activity using the gel shift assay. The strain carrying pYFJ84 had high levels of AasS activity, whereas the control strain carrying vector pET16b was devoid of activity (data not shown), thereby demonstrating that the open reading frame encoded *V. harveyi* acyl-ACP synthetase. Large-scale extracts were then prepared, and the enzyme was purified by nickel chelate chromatography using standard methods (Experimental Procedures). This procedure gave milligram amounts (55 mg from a 500 mL culture) of an essentially pure protein (Figure 4) of high activity. Although Fice et al. (*13*) reported that AasS purified to homogeneity from crude extracts of *V. harveyi* was extremely unstable, our purified His-tagged enzyme was stable and exhibited no detectable loss of activity after being stored for several months at −80 °C. The purified His-tagged AasS preparations had specific activities of ~2.5 μmol of myristoyl-ACP formed per minute per milligram of protein as measured by the radioactive assay and the calculated (*34*) extinction coefficient of AasS. This specific activity is comparable to that obtained for *E. coli* Aas (*8*) but only approximately one-half of that reported for AasS purified to homogeneity from *V. harveyi* (*13*). How-

A.

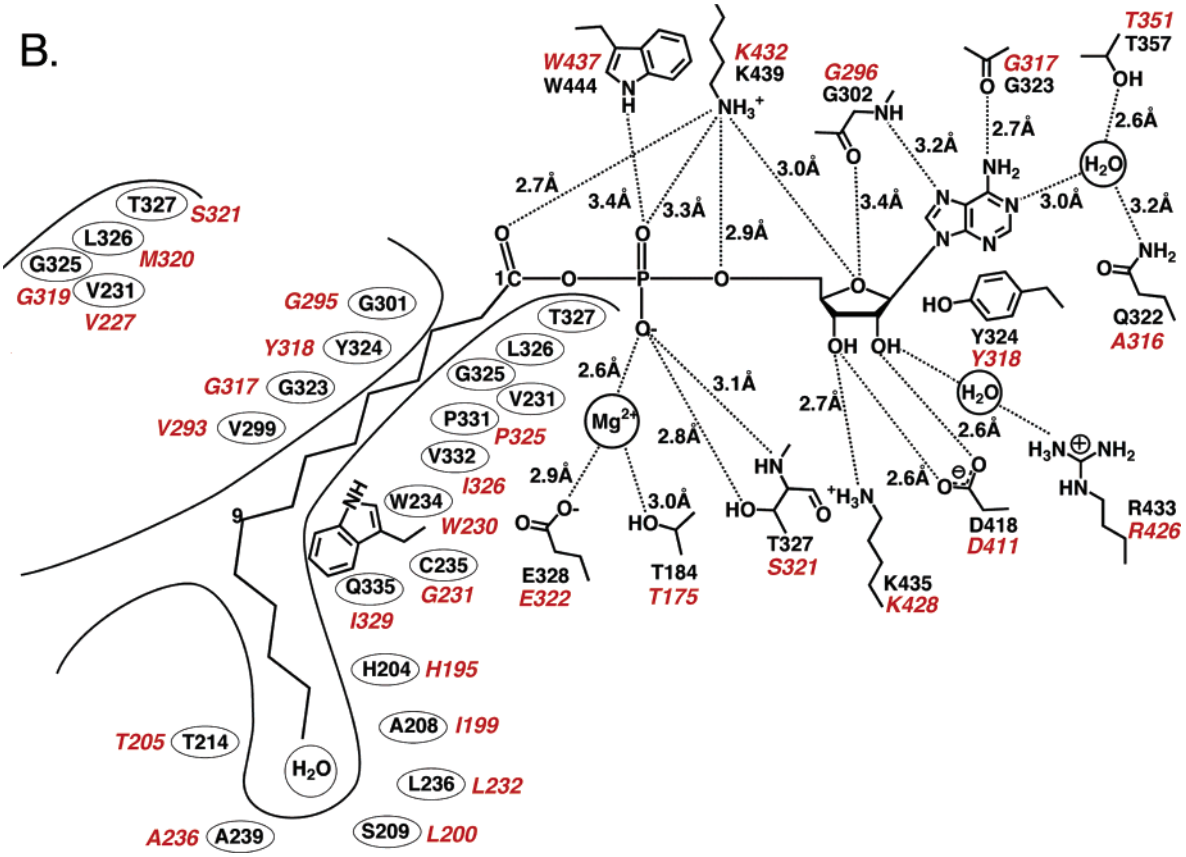

FIGURE 2: Residue alignments and a cartoon of the *Thermus* acyl-CoA synthetase active site with AasS residues superimposed. (A) Clustal W alignments of AasS (top line) with (in descending order) *E. coli* AasS (C-terminal half), *Thermus* acyl-CoA synthetase, and two *E. coli* acyl-CoA synthetases, FadD and FadK. The circles denote residues conserved between AasS and the *Thermus* acyl-CoA synthetase that are not conserved in the other proteins. The bars over the alignments denote conserved motifs. The black bar is the A3 motif; the dashed red bar is the A motif, and the thin red bar is the FACS motif within which (thick red bar) is the A8 motif. The FACS domain also includes the sequences believed to be characteristic of medium chain acyl-CoA synthetases (*53*). (B) Conservation of active site residues of the *Thermus* acyl-CoA synthetase with *V. harveyi* AasS. The figure is modified from Figure 5C of ref *32* with permission. The *Thermus* acyl-CoA synthetase residues are circled, whereas the residues labeled in red italics are those of *V. harveyi* AasS. The inset is the region that interacts with the carboxyl carbon and carbons 2 and 3 of the acyl chain where crowding precluded insertion of the AasS residues.

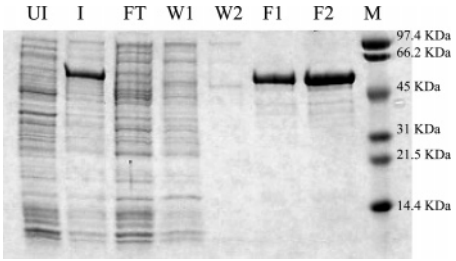

FIGURE 3: Purification of N-terminally His-tagged *V. harveyi* AasS. Fractions from the Ni−NTA purification were dialyzed against AAS buffer and loaded on a 12% SDS−PAGE gel. Abbreviations: UI, crude extract of an uninduced culture; I, crude extract of an induced culture; FT, flow-through; W1 and W2, wash fractions 1 and 2, respectively; F1 and F2, combined His-tagged AasS fractions 1 and 2, respectively; and M, Bio-Rad prestained protein molecular mass markers (low range).

ever, only 3 μg of protein remained after the final step of the purification from *V. harveyi*, and hence, determination of the specific activity seems likely to have been problematic.

V. harveyi AasS Has a Narrow Protein Substrate Specificity. The fatty acyl chain length specificity of AasS was reported previously (*21*) and was further confirmed and extended in this work. Although we routinely use *E. coli* ACP as the substrate [which is as active as *V. harveyi* ACP (*35*)] in the AasS reaction, we were also interested in whether AasS could acylate ACPs from other species. Several ACPs from different species, including *Aquifex aeolicus*, *Bacillus subtilis*, *L. lactis*, and the *B. taurus* (bovine) mitochondrial ACP (*36*), have been cloned (N. DeLay and J. E. Cronan, manuscript in preparation). These ACPs have been tested for their abilities to be modified by phosphopantetheine attachment catalyzed by *E. coli* AcpS (N. DeLay and J. E. Cronan, manuscript in preparation) and to be converted from the holo form to the apo form by the newly characterized *E. coli* ACP phosphodiesterase (AcpH) (*18*). The various different ACPs were expressed from a plasmid-based pBAD promoter, and cell-free crude extracts of these strains were used as the acyl acceptors in the AasS assay. Prior work had shown that *L. lactis* ACP was not phosphopantetheinylated by *E. coli* AcpS, and the mitochondrial ACP of *B. taurus* was only poorly modified (*18*) (N. DeLay and J. E. Cronan, manuscript in preparation). Therefore, strains carrying these two genes were additionally transformed with pNRD136 which expresses Sfp, a phosphopantetheinyl transferase of broad substrate specificity (*37*). For each ACP species, AasS reactions were performed with [1-^{14}C]octanoic acid. Labeled bands were observed with *A. aeolicus* ACP [the two labeled bands are consistent with the prior finding

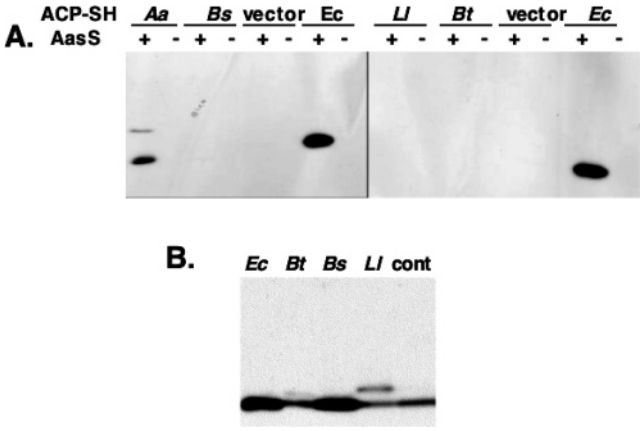

FIGURE 4: Protein substrate specificity of *V. harveyi* AasS. (A) As described in Experimental Procedures, *E. coli* strain DH5α harboring plasmids encoding different ACP species were induced and the cell-free crude exacts were used as the substrate for attachment of [1-^{14}C]octanote using purified His-tagged AasS in the AasS assay: (+) AasS treatment (−) no enzyme treatment. A positive control was conducted with *E. coli* ACP, and a negative control was performed with the crude extract of the strain harboring vector pBAD322. The reactions were analyzed by electrophoresis using 20% gels containing 0.5 M urea, and the dried gels were exposed to X-ray film for 1 week. Abbreviations: Aa, *A. aeolicus*; Bs, *Ba. subtilis*; Ll, *L. lactis*; and Bt, *B. taurus* (bovine) mitochondrial ACP. (B) Several of the same plasmids used in panel A were introduced into a *panD* strain that also expressed the nonspecific 4′-phosphopantetheine transferase, Sfp. The cultures were grown, and a portion of each was labeled with β-[^{3}H]alanine, a pantetheine precursor (Experimental Procedures). Extracts of the unlabeled cultures were then prepared and used to replace ACP in the AasS reaction. The AasS assays were then normalized to the amount of β-[^{3}H]alanine-labeled ACP and were performed with [^{3}H]octanoate to give increased sensitivity over that given by [1-^{14}C]octanote in panel A (Experimental Procedures). The abbreviations are as in panel A except for that of a control culture of the strain carrying the *E. coli* ACP grown in the presence of glucose to repress the *araBAD* promoter which is denoted as cont. The gel was a 4 to 20% gradient gel run without denaturant (*36*), and detection was by fluorography using preflashed film (*54*) at −80 °C for 8 days.

of multiple forms of *A. aeolicus* holo-ACP using the same expression system (*18*)] and *E. coli* ACP, but not with the three other ACP species (Figure 4A). However, staining of the gels showed that, unlike the other proteins, the *Ba. subtilis* ACP was very poorly expressed in the experiment depicted in Figure 4A. We repeated the experiment using a host strain that carried both an Sfp-encoding plasmid and a *panD* mutation that permitted labeling of the ACP prosthetic group with tritiated β-alanine, a 4′-phosphopantetheine precursor. We also increased the specific activity of the fatty acid substrate. Cultures of each strain were prepared in arabinose medium containing 4 μM β-alanine, and a small sample of each was moved to a tube containing 45 μCi of β-[3-^{3}H-(N)]alanine (60 Ci/mmol). The large and small cultures were then incubated with shaking in parallel and harvested when growth ceased due to β-alanine limitation. Lysates were prepared, and those from the small radioactive cultures were processed to separate protein-bound label from unincorporated label with CoA and its precursors and thereby give the levels of holo-ACP in each extract. The nonradioactive large extracts were then used as the ACP source in a [^{3}H]-octanoate AasS assay normalized to the holo-ACP level of each extract by use of the values from the parallel β-[3-^{3}H]-alanine-labeled cultures. The *Ba. subtilis* ACP (which was well-expressed and efficiently phosphopantetheinylated in this experiment) was as efficiently octanoylated as *E. coli* ACP, whereas octanoylation of the *L. lactis* ACP was very weak and that of the bovine mitochondrial ACP only barely detectable. We also treated these extracts with a mixture of hydroxylamine and DTT to remove any acyl groups that might block the 4′-phosphopantetheine thiol group (*38*). This treatment gave no increase in the level of octanoylation for any of the ACPs of Figure 4B. From these experiments, it seems that AasS may not be a generally useful reagent for the detection of ACP-like proteins or for synthesis of acyl derivatives of diverse ACPs. Indeed, the pattern of acylation of the various ACPs is very similar to those of AcpS (N. DeLay and J. E. Cronan, manuscript in preparation) and AcpH (*18*), the enzymes that attach and remove the ACP prosthetic group, respectively. Since in the case of AcpS, its cocrystal structure with ACP shows that the interactions are primarily with helix II of ACP (*39*), it seems likely that AasS also recognizes helix II.

Biological Function of V. harveyi aasS. The *aasS* of *V. harveyi* B392 is a unique gene that has (thus far) been found only in this organism. The closest relative to *V. harveyi* for which a complete genome sequence is available is *V. parahaemolyticus* (*40*). Indeed, the DNA sequences upstream and downstream of *aasS* are closely homologous to *V. parahaemolyticus* open reading frames VPA0349 and VPA0356, respectively, suggesting that *aasS* may have replaced an ABC-type transporter (or vice versa) during evolution of the *Vibrio* species. We were interested in the biological function of AasS, especially since that substrate of *V. harveyi* luciferase requires a fatty acid derivative, *n*-tetradecanal, for light production (*41*). We therefore disrupted the *aasS* gene in a wild-type strain of *V. harveyi*, strain B392, confirmed the disruption events (Figure 5), and studied the properties of the engineered mutant strains. It should be noted that the open reading frame downstream of *aasS* (encoding the VPA0356 homologue) is encoded on the opposite DNA strand, and thus, the *aasS* disruption alleles should have no effect on expression of downstream genes. Moreover, insertion of the kanamycin resistance cassettes introduced several copies of each of the three nonsense codons into the *aasS* reading frame (and a strong transcription terminator in one case), thus precluding the function of the disrupted *aasS* genes. Three different strains carrying disruptions made using somewhat different kanamycin cassettes were tested and found to be indistinguishable. All three strains grew normally and exhibited levels and kinetics of bioluminescence identical to that of the wild-type strain during growth in liquid culture and on solid media (data not shown). The *aasS* disruption strains were assayed for AasS activity, and all were found to retain low levels of activity, ∼5% of that of the wild-type strain (a rough estimate since the residual response was not linear with protein concentration). This result was not unexpected since a reproducible second small peak of activity in addition to AasS was reported in the ion exchange chromatographic purification of AasS from *V. harveyi* extracts (*13*). Moreover, *V. fischeri* was reported to contain acyl-ACP synthetase activity (*12*), although its genome sequence (*42*) lacks a close homologue of *V. harveyi* AasS. Byers (*16*) also reported that, unlike *E. coli*, *V. harveyi* was able to elongate exogenously supplied short chain fatty acids to longer chains that were subsequently

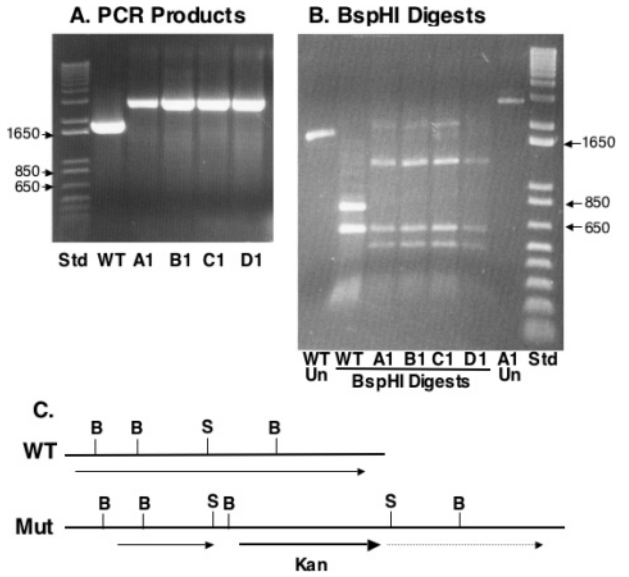

FIGURE 5: PCR analyses of an *aasS::kan* disruption allele. In each panel, the wild-type strain and four independent strains resistant to kanamycin and sucrose, but sensitive to gentamycin, are shown. (A) PCR products obtained using primers that anneal just outside the coding region and thus fail to anneal to the plasmid used for disruption. The *aasS* region of *V. harveyi* wild-type strain B392 gives the expected band of 1752 bp, whereas the *aasS::kan* disruption strains give the expected 2701 bp fragment. Strain CY1719 is strain A1 of panels A and B. The PCR product of the wild-type strain was cleaved to fragments of 183, 216, 765, and 589 bp (left to right in the top map of panel C) by BspHI as expected, whereas digestion of the PCR products of the *aasS::kan* disruption strains gave fragments of 183, 216, 462, 1255, and 580 bp (left to right in the bottom map of panel C). (C) The maps of the PCR products are drawn to scale. Abbreviations: B, BspHI; S, SmaI; and Std, standard. The standard is the 1 kb Plus DNA ladder (Invitrogen) which consists of 12 bands in 1000 bp increments ranging in size from 1 to 12 kb with a 1650 bp fragment (doublet) and fragments from 100 to 850 bp.

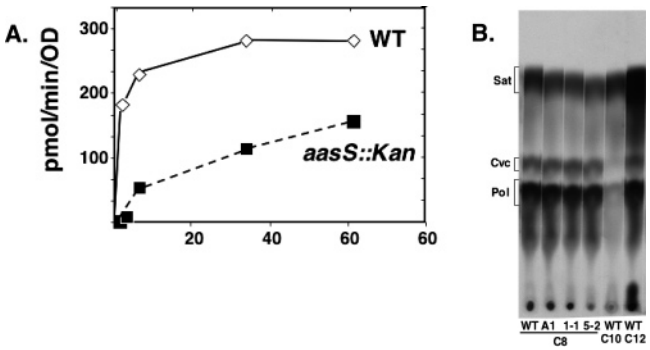

FIGURE 6: Incorporation of 1-^{14}C-labeled fatty acids into the phospholipids of *V. harveyi* wild-type strain B392 and its *aasS::kan* derivatives. (A) Wild-type strain B392 (denoted WT) and its *aasS::*Ω*Km* derivative strain CY1723 were grown in the medium of Byers (*16*), and exponentially growing cultures were labeled with sodium [1-^{14}C]octanoate at the concentrations that are shown. The labeling times were varied with concentration such that <10% of the added octanoate was incorporated and the rate of incorporation was linear with time. Labeling was terminated by addition of 3.75 volumes of a chloroform/methanol mixture (1:2, by volume). The phospholipids were obtained in the chloroform phase formed by addition of 1.25 volumes each of chloroform and 1 M KCl. The octanoate that had partitioned into the chloroform phase was removed by thin-layer chromatography (A) or by base-catalyzed transesterification of the phospholipid acyl chains to their methyl esters as in panel B. The values are given in terms of the absorbance of dilutions the *V. harveyi* cultures at 660 nm using the conversion factor of 1 OD unit equals 10^9 cells/mL (*15*, *16*). (B) Argentation thin-layer chromatographic separation of fatty acid methyl esters derived from the phospholipids of cells of wild-type (WT) *V. harveyi* strain B392 and three *aasS::kan* disruption alleles (A1, 1-1, and 5-2 which are strains CY1719, CY1723, and CY1724, respectively). In lanes 1−4, the strains were labeled with 5.5 μM sodium [1-^{14}C]octanoate (55 mCi/mmol), whereas in lanes 5 and 6, strain B392 was labeled with either 2.5 μM sodium [1-^{14}C]-decanoate (55 mCi/mmol) (lane 5) or 5.5 μM sodium [1-^{14}C]-dodecanoate acid (55 mCi/mmol) (lane 6). Labeling was conducted essentially as reported by Byers (*16*) except that labeling commenced at cell concentrations of $\sim 4 \times 10^8$ cells/mL and proceeded for 25 min. Reverse phase thin-layer chromatography of these same methyl ester samples showed that all of the exogenously added acids that were incorporated had been elongated to chain lengths of 14−18 carbons except that a portion of the dodecanoate acid was incorporated without elongation. Abbreviations: Sat, saturated fatty acid methyl esters; Cvc, methyl *cis*-vaccenate (*cis*-11,12-octadecenoate); and Pol, methyl palmitoleate (*cis*-9,10-hexadecenoate).

incorporated into phospholipids and lipid A. Since exogenous fatty acids entered the fatty acid biosynthesis cycle, they must have been converted to their acyl-ACP derivatives, and thus, Byers (*16*) made the reasonable prediction that AasS could have a role in this process. We have used our *aasS* disruption strains to test this proposal and found that AasS does play a role in the elongation of octanoic acid to chain lengths sufficient for incorporation into phospholipids.

We studied utilization of octanoic acid because it is an excellent AasS substrate (*11*) and is not a normal component of the phospholipids. Moreover, Byers (*16*) had shown that *V. harveyi* B392 converts exogenous octanoate to octanoyl-ACP that is then elongated to 16 and 18 carbon fatty acyl-ACP species by the fatty acid synthetic pathway. These long acyl chains are then incorporated into membrane phospholipids, mainly phosphatidylethanolamine and phosphatidylglycerol (*16*) (data not shown). Our preliminary results suggested that the *aasS::kan* strains had a lower affinity for octanoate than the wild-type strain, and thus, incorporation was assayed over a >100-fold range of octanoate concentrations (Figure 6A). Although the rate of incorporation of octanoate into the phospholipids by the wild-type strain was relatively insensitive to octanoate concentration, the incorporation rates of the mutant strains were quite sensitive to octanoate concentration with the rates dropping off rapidly at low concentrations. Hence, although the mutant strains exhibited only modest deficiencies at high octanoate concentrations, the incorporation rates of the mutant strains were <10% of that of the wild-type strain at the lower concentrations. The biphasic shape of the saturation curve given by the mutant strain (Figure 6A) suggests that the loss of AasS uncovers two other incorporation systems. One of these could be the enzyme responsible for the residual acyl-ACP synthetase activity in the *aasS::kan* strains and for the minor peak reported by Fice and co-workers (*13*), whereas the other enzyme could be the putative acyl-CoA::ACP transacylase activity invoked by Shen et al. (*43*) to explain formation of shorter chain acyl-ACPs from exogenous tetradecanoic acid. Whatever the nature of the incorporation machinery that remains in the *aasS::kan* strains, the incorporation pattern was similar to that of the wild-type strain (Figure 6B). When the wild-type strain and the three *aasS* disruption strains were labeled with [1-^{14}C]octanoic acid and conversion of the labeled acid to phospholipid long chain fatty acid moieties

was assayed by thin-layer chromatography using argentation (Figure 6B) and reverse phase (data not shown) plates, we found that the strains carrying *aasS::kan* disruptions efficiently incorporated [1-^{14}C]octanoic acid into long chain saturated and unsaturated phospholipid acyl chains. As previously reported by Byers (*16*), this cannot be attributed to β-oxidation of the octanoate to acetyl-CoA and subsequent utilization of the resulting [1-^{14}C]acetyl-CoA because when wild-type cells were labeled with [1-^{14}C]decanoic acid very little label was found in the unsaturated fatty acid species (Figure 6B) (reverse phase thin-layer chromatography showed that all of the label was present in elongation products). However, in contrast to a prior report (*16*), we found that incubation with [1-^{14}C]dodecanoic acid gave labeled unsaturated species in addition to the saturated species previously reported (Figure 6B). This was not unexpected because Shen and Byers (*43*) reported β-oxidation of exogenously supplied tetradecanoic acid, and the original work shows traces of unsaturated species in cells labeled with [1-^{14}C]dodecanoic acid (*16*). We suspect that the differing results are due to induction of β-oxidation by fatty acids of $\geq$12 carbons, whereas shorter chains fail to induce or induce weakly. This is the case in *E. coli* where the induction pattern is due to the differing binding affinities of short and long chain acyl-CoA species for the FadR transcription factor (*44*). It seems likely that *V. harveyi* contains a FadR protein similar to that of *E. coli* since the FadR of *Vibrio cholerae* has a pattern of acyl-CoA binding similar to that of the *E. coli* protein (*45*). The experiments of Byers were carried out at very high cell densities ($>10^9$ cells/mL) which might have inhibited induction (or function) of β-oxidation, whereas we labeled cultures at densities of $2-5 \times 10^8$ cells/mL. Moreover, we studied wild-type strain B392, whereas many of results obtained in the Byers laboratory used strain M17, a dark mutant derived from strain B392.

V. harveyi B392 may possess such a diversity of mechanisms to utilize short and medium chain fatty acids because planktonic *V. harveyi*, an organism found in very low quantities in almost all oceans of the world, subsist on dissolved organics. However, *V. harveyi* is also a pathogen and is the cause of serious losses in marine aquaculture of fish and invertebrates. It should be noted that the presence of AasS does not seem to be an invariant property of *V. harveyi* since we have found that *V. harveyi* strains BB120 and BB720 of Bassler and co-workers (*46*) contain no detectable AasS activity (data not shown). Moreover, the extant database (www.genome.wustl.edu/genome.cgi) of the ongoing *V. harveyi* strain BB120 genome project contains no sequences that match *aasS*, although a sequence closely matching that found immediately downstream of *aasS* is located within a large block of contiguous sequence. The differing AasS contents of the *V. harveyi* strains are not completely unexpected because the *V. harveyi* designation is known to include a phenotypically diverse set of organisms (*47*, *48*). Indeed the taxonomy of this and many other *Vibrio* species is the subject of much ongoing debate (*49*, *50*).

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